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Carl E. Pedersen, Jr., et al

Army Medical Research Institute of Infectious Diseases
Frederick, Maryland

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CARL E. PEDERSEN, JR., DONALD R. SLOCUM, AND DAVID M. ROBINSON

Institute of Infectious Disease[illegible] Frederick, Maryland 21701

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CARL E. PEDERSEN, JR., DONALD R. SLOCUM, AND DAVID M. ROBINSON
U.S. Army Medical Research Institute of Infectious Diseases, Frederick, Maryland 21701

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Small- and large-plaque variants of a Florida strain (Fe 3-7c) of Venezuelan equine encephalomyelitis virus were studied in vivo and in vitro. The small-plaque variant was less virulent in mice, hamsters, and guinea pigs than the large-plaque variant. The variants could be distinguished by calcium phosphate chromatography. The implications of plaque variants within a mixed virus population are discussed.

Venezuelan equine encephalomyelitis (VEE) virus endemic to the United States was first isolated by Chamberlain et al. in 1963 (4) from *Culex* mosquitoes collected in the Florida Everglades. The prototype strain, Fe 3-7c, has been placed in antigenic subgroup II on the basis of kinetic hemagglutination inhibition serology (21). This virus has been suggested as a candidate for development of a live vaccine (10) as it is considered to be avirulent for horses and appears to confer protection equivalent to that elicited by the TC-83 strain of VEE (1). We have detected two distinct plaque types in the Fe 3-7c virus population propagated in duck embryo cells (DEC). This report deals with studies conducted with large-plaque (LP) and small-plaque (SP) variants derived from the parent Fe 3-7c virus (PV).

MATERIALS AND METHODS

Virus strain. The seed stock of Florida virus (Fe 3-7c) was kindly provided by R. Shope of the Yale Arbovirus Research Unit, Yale University, New Haven, Conn. This virus strain, which contained mixed plaque types, was a 10% suckling mouse brain preparation which had been passed five times in suckling mice via the intracerebral (ic) route.

Virus assay. Plaque assays were performed essentially as described by Dulbecco and Vogt (6). Serial 10-fold dilutions of virus were prepared in phosphate-buffered saline containing 1.0% normal rabbit serum, penicillin (100 units/ml), and streptomycin (50 μg/ml) (PBS). Virus inocula were added to monolayers of duck (DEC) or chicken embryo cells (CEC) in flasks and allowed to adsorb for 1 hr at 37 C. The cells were overlaid with medium 199 containing Earle balanced salt solution, 3.0% calf serum, 1.0% lonagar, penicillin (100 units/ml), and streptomycin (50 μg/ml). After incubation at 37 C for 48 hr the cells were overlaid with 2.0 ml of neutral red (0.1 mg/ml) in Hanks balanced salt solution to facilitate reading of plaques. Virus titers are expressed as plaque-forming units (PFU) per ml. In vivo titrations were performed by inoculating suckling mice ic with 0.03 ml of a virus dilution. Mice were observed daily for 10 days for signs of illness or death. The median lethal dose was calculated by the method of Reed and Muench (14).

Plaque selection. Cell cultures with agar overlay were prepared as described above. After 48 hr, distinct plaque types were aspirated with a curved Pasteur pipette. The cells within the plaque and the agar immediately above the plaque were dispersed in 1.0 ml of PBS. Five plaques were selected at each cloning step, and each plaque was titrated separately for the presence of LP or SP variants. The final pools of SP and LP variants were prepared from the third consecutive subclone of the appropriate variant.

Animals. Golden Syrian hamsters (85 to 95 g) were obtained from the Lakeview Hamster Colony, Newfield, N.J. Hartley strain guinea pigs (250 to 300 g) were obtained from the West Jersey Biological Supply Farms, Wenonah, N.J. Swiss albino mice (CD-1 strain) were obtained from the Charles River Breeding Labs., Wilmington, Mass. Animals were inoculated intraperitoneally (ip) or subcutaneously (sc).

Chromatographic technique. The brushite form of calcium phosphate ($CaHPO_4 \cdot 2H_2O$) was prepared from 0.5 M $CaCl_2$ and 0.5 M Na_2HPO_4 as described by Burness (3). The gel was stored at 4 C in 0.005 M phosphate buffer, pH 7.4, until used. Columns were prepared by pouring 75 ml of the slurry into a 2.0 by 50.0 cm glass column. After a 15-min settling period the gel was washed with 0.005 M phosphate buffer until the surface position was constant (routinely 25-ml packed volume). The column was checked for channeling or skewing with a band of bromocresol purple, and the amount of eluate collected before the first drop of dye appeared was considered the void volume. A 1-ml amount of concentrated virus (ca.

10^8 PFU/ml) was then added to the gel, and a linear gradient of elution buffer (0.1 to 0.6 M phosphate buffer, pH 7.4) was applied. The flow rate of the column was 0.5 to 1.0 ml/min, and 5.0-ml fractions were collected. The infective virus titer of each fraction was determined by plaque assay.

Serologic tests. Hemagglutination inhibition tests were performed by the method of Clarke and Casals (5). Neutralization tests were performed essentially as described by Dulbecco et al. (7). Gel diffusion tests were performed by the method of Ouchterlony (13) using 0.75% agarose buffered with sodium Veronal, pH 8.6, ionic strength 0.075.

Immune sera. Guinea pig immune serum was obtained 21 days after ip injection of the Fe 3-7c PV, or LP or SP variant.

RESULTS

Selection of plaque variants. The seed lot of Fe 3-7c contained 5.3×10^8 PFU/ml. Plaques varied from 1.5 to 5.0 mm after 48 hr on DEC. Approximately 4.0% of the population consisted of SP variants (< 2 mm). Upon sequential passage in DEC, the SP population increased to 42% by the third passage, and the titer increased to 4.0×10^9 PFU/ml. Plaque variants selected from this DEC subpassage were picked, and each was carried through three cloning steps. Figure 1 illustrates the Fe 3-7c PV and cloned SP and LP variants.

The SP variants ranged from 1.4 to 2.0 mm after 48 hr and 1.9 to 3.4 mm after 72 hr on DEC. LP variants were not detected in any passage of the SP variant. The average virus yield was approximately 1×10^9 PFU/plaque selected. The SP pool utilized in this study was harvested from CEC and contained 4.0×10^9 PFU/ml. No LP variants were detected in the pool. Individual plaques produced by this SP clone did not have well-defined margins but were generally very clear throughout the plaque.

The LP variants ranged from 4.0 to 5.2 mm after 48 hr and 5.5 to 10.9 mm after 72 hr on DEC. The average virus yield was approximately 1×10^9 PFU/plaque selected. The LP pool utilized in this study was harvested from CEC and contained 4.1×10^9 PFU/ml. LP virus clones always contained 2 to 4% of the SP variant. The individual plaques induced by the

FIG. 1. *Plaque types of Fe 3-7c on chicken embryo cells (CEC) stained with 1% crystal violet in ethanol. The parental virus (PV) was stained after 48 hr, large plaque (LP) and small plaque (SP) variants were stained after 72 hr of growth in CEC.*

LP clone had very clear centers with slightly more opaque, irregular margins. They were visible by 18 hr postinoculation and measured at least 1 mm in diameter. The plaque size then increased rapidly to 14 mm by the fifth day.

Animal studies. Table 1 shows the results obtained when weanling mice, hamsters, and guinea pigs were inoculated with the PV, and SP and LP variants of Fe 3-7c. The LP variant was the most virulent of the viruses studied and showed a virulence profile very similar to the PV. The SP variant was avirulent for guinea pigs and hamsters; however, some weanling mice were killed by this variant. Immunization with PV, LP, or SP conferred protection to the survivors; however, the protection was not absolute.

In a separate experiment, adult mice were injected intravenously with LP and SP variants. The LP variant killed 7 of 9 mice 10 to 14 days postinoculation. Histopathological examination of the brains from dead mice showed severe, acute, diffuse nonsuppurative meningo-encephalitis with severe neuronal and glial necrosis. Histopathological examination of mice which were not killed by the LP variant showed chronic nonsuppurative meningo-encephalitis with moderate multifocal chronic vasculitis of the brain. In contrast, none of 9 mice injected with the SP variant died. Histopathological examination showed essentially normal mouse brain.

Suckling mice were uniformly susceptible to ic injection with either the LP or SP variants of Fe 3-7c. Although this test host was approximately 10-fold more sensitive than the plaque assay of infective viruses, no differences were observed between the lethality of the LP and SP variants.

Calcium phosphate chromatography. The elution profile of Fe 3-7c (PV) is illustrated in Fig. 2. Two distinct peaks were obtained, and plaques were individually scored as large (>2 mm) or small (<2 mm). The first peak, eluted in the region of low ionic strength, contained predominantly LP virus, whereas the second peak contained predominantly SP virus. We subsequently chromatographed the variants separately, and the results are depicted in Fig. 3 and 4. The peak of LP variant infectivity was eluted from the column by 0.19 M phosphate buffer. The peak of SP variant infectivity was eluted by 0.30 M phosphate buffer. These results are consistent with the experimental chromatography of the mixed parental population.

Serologic studies. Using guinea pig sera we performed neutralization tests with the Fe 3-7c PV and LP and SP variants. The results showed

TABLE 1. *Responses of test animals to inoculation with Fe 3-7c VEE virus and subsequent challenge with virulent (Trinidad) VEE virus*

Fe 3-7c	Virus dose[a] (CEC PFU)	Weanling mice		Hamsters		Guinea pigs	
		% Survivors (no./total)	% Protected[b] (no./total)	% Survivors (no./total)	% Protected[b] (no./total)	% Survivors (no./total)	% Protected[b,c] (no./total)
Parental virus	2×10^5	0 (0/8)		20 (1/5)	100 (1/1)	100 (6/6)	100 (5/5)
	2×10^4	0 (0/8)		20 (1/5)	100 (1/1)	100 (6/6)	80 (4/5)
	2×10^3	0 (0/8)		20 (1/5)	100 (1/1)	100 (6/6)	80 (4/5)
	2×10^2	0 (0/8)		40 (2/5)	100 (2/2)	100 (6/6)	100 (5/5)
	0	100 (8/8)	0 (0/8)	100 (5/5)	0 (0/5)	100 (6/6)	0 (0/5)
Large-plaque variant	2×10^5	0 (0/5)		0 (0/5)		0 (0/5)	
	2×10^4	0 (0/5)		0 (0/5)		0 (0/5)	
	2×10^3	0 (0/5)		20 (1/5)	100 (1/1)	20 (1/5)	100 (1/1)
	2×10^2	0 (0/5)		40 (2/5)	100 (2/2)	80 (4/5)	50 (2/4)
	0	100 (5/5)	0 (0/5)	100 (5/5)	0 (0/5)	100 (5/5)	0 (0/5)
Small-plaque variant	2×10^5	20 (1/5)	100 (1/1)	100 (5/5)	80 (4/5)	100 (5/5)	80 (4/5)
	2×10^4	60 (3/5)	100 (3/3)	100 (5/5)	100 (5/5)	100 (4/4)	100 (4/4)
	2×10^3	60 (3/5)	100 (3/3)	100 (5/5)	80 (4/5)	100 (5/5)	80 (4/5)
	2×10^2	40 (2/5)	100 (2/2)	100 (5/5)	80 (4/5)	100 (5/5)	40 (2/5)
	0	100 (5/5)	0 (0/5)	100 (5/5)	0 (0/5)	100 (5/5)	0 (0/5)

[a] Weanling mice and guinea pigs were inoculated ip with 0.5 ml of the virus. Hamsters were inoculated sc with 0.5 ml of the virus. Sham-inoculated animals were included as challenge controls.

[b] Challenge dose was 10 SMICLD$_{50}$ (median lethal dose to suckling mice via the intracerebral route) given at 21 days.

[c] One guinea pig from each group was exsanguinated before challenge.

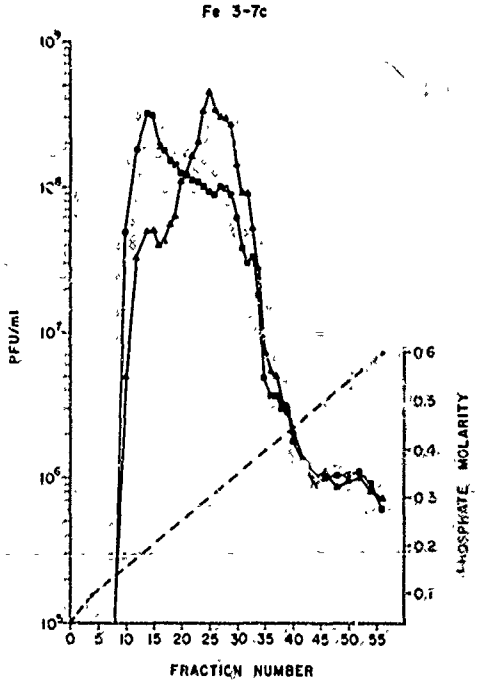

FIG. 2. *Elution pattern of the parental Fe 3-7c virus from calcium phosphate chromatography. Plaques were individually scored as large (>2 mm) or small (<2 mm) after 48 hr on duck embryo cells. Large plaques (■); small plaques (▲).*

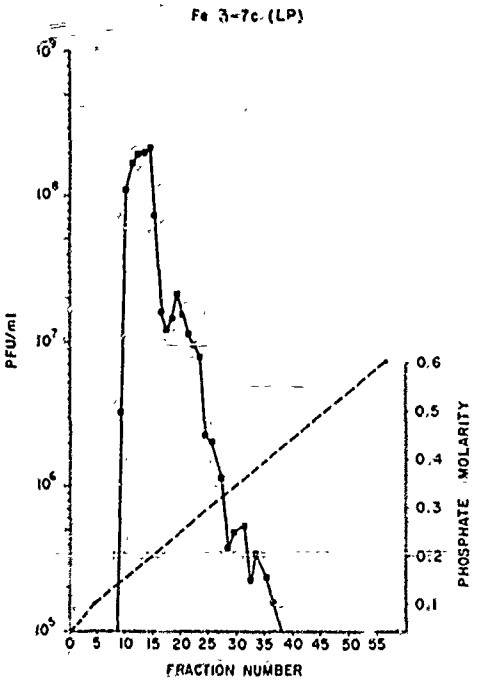

FIG. 3. *Elution pattern of the large-plaque variant from a calcium phosphate column.*

that the LP and SP variants were readily neutralized by sera directed against the PV, LP, or SP virus. Gel diffusion tests demonstrated that the antigens were related; however, we were unable to detect any differences between the plaque variants by this technique.

DISCUSSION

In light of the zoonotic in the United States during the summer of 1971, there is renewed interest in the disease potential of VEE virus. The current vaccine used to prevent infection with VEE virus is the attenuated TC-83 strain derived from the Trinidad strain of VEE virus isolated from the brain of a donkey (19). However, certain objections concerning the use of this vaccine have been based on the possibility of reversion of TC-83 to the virulent parental form of the virus (1, 10). In this light, it has been suggested that a Florida strain of VEE (Fe 3-7c, endemic to the United States) might be used in place of TC-83 (10).

Zarate and Scherer (22) found Florida strains of VEE virulent for hamsters; however, the incubation period was longer than that seen with Mexican VEE strains. In contrast, the TC-83

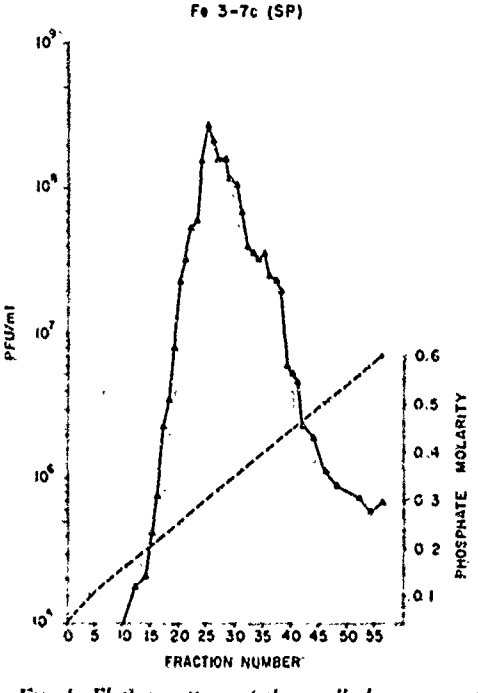

FIG. 4. *Elution pattern of the small-plaque variant from a calcium phosphate column.*

vaccine strain kills only 15 to 20% of inoculated hamsters (F. E. Cole, Jr., *personal communication*). Horses immunized with Fe 3-7c developed a transient viremia, but the reported effects appeared to be less severe than in horses experimentally inoculated with TC-83 (10).

Other investigators have observed variations in the plaque size of VEE (8, 9, 12), and Schaffer and Scherer (16) have recently reported a range of 1.4 to 6.9 mm in the plaque size of the Fe 4-71k strain of Florida VEE. We found a heterogeneity of plaque sizes in the Fe 3-7c VEE strain (10% suckling mouse brain), and with subpassage in cell culture the ratio of SP to LP increased. This effect has been reported for Western equine encephalitis virus (11), [illegible] virus (20), and foot and mouth disease virus (7). The shift to small plaque character in cell culture is felt to be associated with an increased sensitivity to inhibitors present in agar (18).

Cloning the plaque variants showed that each had distinct biological and chemical characteristics. The LP variant was uniformly virulent for weanling mice and was highly lethal for hamsters, guinea pigs, and adult mice. In contrast, the SP variant was avirulent for hamsters, guinea pigs, and adult mice and less virulent for weanling mice than either PV or the LP variant. Animals which survived the initial inoculation of Fe 3-7c viruses were generally protected from the lethal effects of a virulent (Trinidad) strain of VEE challenge virus. We feel that the presence of LP variants in the PV virus population (58%) probably contributed to the deaths observed with hamsters and weanling mice. This does not explain the resistance of guinea pigs to the Fe 3-7c PV; however, species specificity might permit the SP variant to multiply preferentially or interfere with the LP virus and mask the virulence of the LP variant.

Chromatographic experiments reinforced the findings of biological differences between the variants. These differences may be expressed by the charge on the viral membrane which would account for the individual elution profiles of the variants on calcium phosphate columns. Other investigators (2, 16) have found that LP and SP variants can be distinguished by brushite chromatography, and in these systems the LP variant was eluted at a lower ionic strength of buffer than the SP variant, as was the case in our study.

We have found that the SP variant of Fe 3-7c is relatively avirulent and confers protection to test hosts. Although our studies indicate that the SP variant of Fe 3-7c would offer no advantage over the TC-83 vaccine strain currently in use, the SP variant is less virulent in hamsters and therefore might deserve trial in horses to learn whether it might produce less overt illness than TC-83. In addition, the use of either the PV or the LP variant of Fe 3-7c would certainly be contraindicated based on our results with animal model systems. It may be that the characteristics applied to a mixed virus population containing plaque variants are attributable to the percentage of LP or SP within that population. Therefore, if a given virus is to be considered as a candidate for an attenuated vaccine, the biologic and genetic characteristics of plaque variants within that strain should be investigated.

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